British Journal of Nutrition (2003), **90**, 503–506
DOI: 10.1079/BJN2003931

Short communication

Quantification of unlabelled non-haem iron absorption in human subjects: a pilot study

Jack R. Dainty*, Mark A. Roe, Birgit Teucher, John Eagles and Susan J. Fairweather-Tait
Institute of Food Research, Colney Lane, Norwich NR4 7UA, UK

(Received 15 November 2002 – Revised 17 April 2003 – Accepted 10 May 2003)

A method for measuring unlabelled Fe absorption has been investigated in a pilot study using a simple mathematical model. The metabolism of newly absorbed Fe can be approximated as a single-compartment model with the sampled compartment being the plasma pool. Five female volunteers (aged 30–55 years) were recruited to participate in the pilot study. After a 10 mg oral dose of unlabelled ferrous sulfate, the change in plasma Fe concentration over the following 6 h was used to estimate the quantity of absorbed Fe from the mathematical model. To assess the accuracy of the new technique, a 1 mg oral dose of ^{57}Fe-labelled iron sulfate was given simultaneously with a 225 μg intravenous dose of ^{58}Fe as iron citrate. The plasma appearance of the labelled Fe was used to estimate the absorption of the oral label from the traditional area under the curve method. There was no significant difference ($P=0{\cdot}61$) between the geometric mean absorption of the unlabelled (19 (−1 SD 12, +1 SD 28) %) and the labelled Fe (17 (−1 SD 10, +1 SD 29) %). These initial results are encouraging, but further work needs to be undertaken with smaller doses, as typically found in meals. The effect of diurnal variation in serum Fe concentration on the estimation of unlabelled Fe absorption needs further assessment.

Iron: Mathematical models: Kinetics

Fe absorption has traditionally been measured from the appearance of radio- or stable-isotope-labelled Fe doses in either blood or faecal samples. Newly absorbed labelled Fe is incorporated into erythrocytes and a single blood sample taken 14 d after the oral dose is sufficient to enable an estimate of Fe absorption to be made. Alternatively, a complete faecal collection can be undertaken for approximately 10 d and the unabsorbed label measured directly. Both of these techniques require radio- or stable-isotope-labelled Fe in the test meal because the unlabelled Fe that naturally occurs in foods cannot be distinguished from endogenous Fe once it is inside the body or in faeces. Almost all non-haem Fe bioavailability studies have used extrinsic labels, based on the assumption that the Fe from the label will form a common pool in the gut with non-haem Fe from unlabelled dietary sources and be absorbed identically. Generally, the UK diet contains a mixture of haem (10 to 15 %) and non-haem Fe (85 to 90 %) and to quantify dietary Fe absorption, a method is required that can measure the absorption of both forms simultaneously. If this method can be applied to single meals with an Fe content of about 5 mg then the total quantity of unlabelled Fe absorbed from a meal can be determined without the need to use extrinsic or intrinsic isotope labels.

Previous authors (Gonzalez *et al.* 2001) have attempted to use the increase in serum Fe concentration to estimate unlabelled Fe absorption. The present paper is the first, however, to describe the development of a method for estimating unlabelled non-haem Fe absorption with the use of a simple compartmental model. Compartmental modelling is a well-established technique for estimating fluxes and pool sizes without the need for invasive sampling of body tissues (Jacquez, 1996). Its use in Fe metabolism is based on radioisotope work, published mainly in the 1960s, which resulted in the development of complicated multicompartmental models (for example, Pollycove & Mortimer, 1961). In the present study a simpler compartmental approach has been taken that attempts to model the short-term metabolism of Fe following a single unlabelled dose of ferrous sulfate. The techniques employed are similar to pharmacokinetic drug trials

Abbreviations: AUC, area under the curve; iv, intravenous.

* **Corresponding author:** Dr Jack Dainty, fax +44 1603 507723, email jack.dainty@bbsrc.ac.uk

where single-compartment models are often the best method for the approximate estimation of parameters such as drug half-life, turnover time and fractional absorption (Fox *et al.* 1993). A technique for measuring labelled Fe absorption, the plasma area under the curve (AUC) method (Barrett *et al.* 1994), is used to evaluate the potential of the compartmental approach.

Materials and methods

Subjects

Five female volunteers aged 30–55 years took part in the study. All were in apparent good health as assessed by biochemical screening (full blood count, urea, electrolytes, liver enzymes, glucose and cholesterol) and were Fe replete (haemoglobin >120 g/l). All subjects gave written informed consent and the Norwich District Ethics Committee approved the study.

Preparation and administration of iron solutions for oral and intravenous use

Labelled ferrous sulfate for oral use was prepared from elemental ^{57}Fe (95 % ^{57}Fe; AEA Technology, Didcot, Oxon, UK) using a previously developed method (Fox *et al.* 1998). Iron citrate for intravenous (iv) use was prepared by dissolving 22 mg ^{58}Fe (92 % ^{58}Fe; Isotec, Saint-Quentin Fallavier, France) in 5 ml concentrated HCl (12 M) in a silica crucible and heating until almost dry. Sodium citrate (20 ml of 0·5 M) was added to the iron chloride, giving a citrate:Fe molar ratio in excess of 20:1, which is required to convert iron chloride to iron citrate. The pH was adjusted to 7·0 with 0·1 M-sodium carbonate and the solution was diluted to 250 ml with water for injection. Iron citrate portions for iv use were checked for sterility by the Norfolk and Norwich Hospital Pharmacy.

Experimental protocol

The five subjects were each given 10 mg Fe as sulfate and 20 mg ascorbic acid. In all subjects, following a 12 h overnight fast, a dose of 225 μg ^{58}Fe as citrate was infused and 5 min later the subject was given 300 ml cola drink containing the 1 mg oral dose of ^{57}Fe and 10 mg unlabelled Fe, both as sulfate. For the duration of the study the subjects were only allowed drinking water. Blood samples (25 ml) were taken at 0, 20, 40, 60, 80, 100, 120, 150, 180, 240, 300 and 360 min after the ingestion of the oral doses and collected into trace element-free vacutainers.

Sample analysis

Total serum Fe was measured in duplicate using Ferrozine (total iron diagnostic kit; Sigma, Poole, Dorset, UK). The manufacturer's stated single measurement precision (CV) is approximately 7 %, which was calculated from a group of four serum pool preparations (range of serum Fe concentration 510–2220 μ/l) assayed on eight occasions. According to our calculations, duplicate analysis of a sample should provide a standard error of the mean of about 40 μg/l for a mean of 1000 μg/l. Recovery of a set of Fe standards (Fe concentrations of 2000, 3000 and 4000 μg/l) were 100, 98 and 101 % respectively. Isotope ratios of ^{57}Fe:^{56}Fe and ^{58}Fe:^{56}Fe were measured by thermal ionisation quadrupole MS (Finnegan MAT, GmbH, Bremen, Germany). The measured precision (% relative SD) for these ratios is approximately 1 % and the method has been described previously (Fairweather-Tait *et al.* 1995). A baseline blood sample was also analysed for plasma ferritin using an in-house ELISA with WHO-certified reference standards.

Calculations

Plasma concentrations of the ^{57}Fe oral dose and the ^{58}Fe iv dose were calculated by solving a set of simultaneous equations generated from the mass spectrometer ratios. The AUC was calculated for appearance of the oral and iv doses in plasma using the trapezoidal method.

With reference to Fig. 1, changes in plasma concentration of Fe after an oral dose ($dose_{oral}$) can be approximated as a single-compartment model provided the measurement period is between 3 and 6 h. This length of time allows for the completion of the absorptive process and a good characterisation of the decay of the plasma concentration. The plasma appearance of Fe from the oral dose is assumed to approximate that of an infusion of rate R over time T. The definition of R is:

$$R = \frac{\text{mass of dose that is absorbed}}{\text{time period for absorption}} = \frac{M}{T} \qquad (1)$$

If V is the apparent volume of distribution and k is the rate constant of elimination from the compartment, then the Fe concentration in the compartment (C) can be approximated as:

$$C = \frac{M}{V.T.k} \cdot (1 - e^{-k.t}) \quad (0 < t < T) \qquad (2)$$

$$C = \frac{M}{V.T.k} \cdot (1 - e^{-k.T}) \, .e^{-k(t-T)} \quad (T < t) \qquad (3)$$

By fitting equations 2 and 3 to the plasma concentration data, M, T and k can be calculated. All fitting was performed in Excel (Microsoft Corporation, 2002) using a standard desktop personal computer. Plasma volume, V,

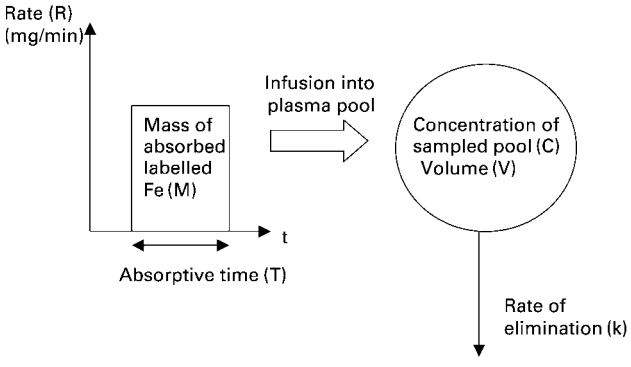

Fig. 1. Schematic overview of the compartmental model.

is estimated from nomograms by knowledge of the volunteer's age and weight (Lentner, 1984).

The fractional absorption can then be calculated according to the equation:

$$\text{Fractional absorption} = \frac{\text{M}}{\text{dose}_{\text{oral}}}. \quad (4)$$

Results and discussion

Table 1 compares calculated percentage Fe absorption, from measurements of plasma appearance of isotopically enriched oral and iv doses (AUC), with serum appearance of orally administered unlabelled Fe, as calculated by the model. Geometric mean absorption measured by plasma appearance of ^{57}Fe using the AUC method (17 (−1 SD 10, +1 SD 29) %) was not significantly different (P=0·61, as calculated by two-tailed, paired, Student's t test) from unlabelled Fe absorption calculated using the model (19 (−1 SD 12, +1 SD 28) %).

There are several criticisms of this new method. The first is the potential error introduced by reliance on an estimate of the plasma volume. The plasma volume estimates were calculated according to the nomograms published in the Geigy tables (Lentner, 1984), and depended on weight, age and sex of the volunteer. An underestimation of plasma volume would lead to an underestimation of the Fe absorption as calculated by the model. For comparison, a different plasma volume method was tried, which was based on body surface area (Hurley, 1975). There was no significant difference between the estimates, and whilst this does not imply that the estimation of plasma volume is accurate, it gives no grounds on which to say that it is not. This remains as a possible small source of error in the model estimate of Fe absorption.

A second source of error is encountered in assuming that a single-compartment model adequately describes the kinetics of newly absorbed Fe. The modelling process can be broken down into two distinct parts: absorption of Fe; plasma Fe kinetics. The absorption process is modelled by assuming that the plasma appearance of newly absorbed Fe has come from the gut via a constant infusion, with a sharply defined start and endpoint. This is a simplification of how Fe is transported across the enterocytes, since the rate of absorption will depend on the quantity of Fe presented to the absorptive cells and the rate of systemic delivery. It would probably be better modelled as a first-order process and this will be investigated in future work. However, as a first approximation, the flux of Fe entering the blood appears to be adequately described as a zero-order process. Any remaining modelling error will be due to the assumptions made concerning Fe kinetics in the plasma. Previous studies have shown that a two- or three-compartment model best describes plasma Fe kinetics in 'normal' subjects studied over a few days (Sharney *et al.* 1965). By simplifying this to a single compartment, it was predicted that the calculation of the elimination rate constant, k, would potentially underestimate the true value. This is due to the Fe 'reflux' from extravascular tissues, which causes Fe, once removed from the plasma, to be returned, thereby increasing the plasma Fe concentration. Since the present study was performed over 6 h, it was thought that the inherent error of assuming a single compartment would be quite small.

Table 1. Comparison of methods used to measure iron absorption from a 1 mg ^{57}Fe-labelled dose and 10 mg unlabelled dose of ferrous sulfate

	Fe absorption (%)		
Volunteer no.	Labelled (AUC)	Unlabelled (Model)	Serum ferritin (μg/l)
1	21	16	16
2	7	10	35
3	28	22	19
4	19	29	24
5	20	23	39
Geometric mean	17	19	25
−1 SD	10	12	17
+1 SD	29	28	37

AUC, area under the curve.

A simulation was performed in order to assess whether this technique is sensitive enough to measure small differences in Fe absorption. The modelling program SAAMII (version 1.1.1; SAAM Institute Inc., Seattle, WA, USA) was used to generate a set of simulated Fe serum concentration data over 360 min. The input conditions for the simulation were that 20·0 % of a 10 mg oral dose of Fe was absorbed into a single compartment of 3000 ml volume (V) over 100 min (T) and the rate of elimination (k) was 0·002/min. These input variables are approximately what were estimated from our volunteers. The simulated output data (serum Fe concentration over 360 min) were then 'overlaid' with an approximate diurnal variation in measured baseline serum Fe concentration estimated from the data of twenty volunteers (Dale *et al.* 2002) by the use of a polynomial function. These output data were processed through our model and it was found that the absorption was now 18·9 %, so the diurnal variation affected the accuracy and led to a systematic error (underestimate) in the 'real' absorption by 5 % of its value (18·9 compared with 20·0 %). No attempt has been made to quantify how random error in the diurnal variation would affect the precision of the absorption estimate but crossover studies are designed to test the same volunteer on more than 1 d and, if the diurnal variation did not remain constant, the precision as well as the accuracy of the estimated absorption would be altered. This has implications for resolving differences in absorption between two or more test meals and will have to be dealt with in a future, more in-depth study.

Finally, using Monte Carlo techniques, random measurement error was added to the data to account for the theoretical variability in the Sigma kit used to measure total serum Fe. The resulting data with randomised error were then generated several times (n 25) and the simulated absorption calculated for each data set using the model described earlier. The average absorption was found to be 19·0 (SD 1·8)% compared with the noise-free simulation of 18·9 % absorption for the simulated data overlaid with a diurnal variation. Two conclusions can be drawn from this

simulation with regard to the use of the single-compartment model for estimating Fe absorption.

First, there is a potential loss of accuracy due to the underestimation of the true Fe absorption because of the decrease in the 'real' baseline serum Fe concentration (diurnal effect). In using the model for the pilot study, it was assumed that the baseline serum Fe concentration was the same throughout the experiment (i.e. the $t = 0$ value). This underestimation will become proportionately larger the lower the dose of Fe and this has implications for extending the use of the model in situations where the Fe dose is less than 10 mg.

Second, the effect of the measurement variation in the Sigma Ferrozine kit leads to an uncertainty of approximately 1·8 % in a simulated absorption of 20 % from a 10 mg dose. For the purposes of the following statistical work, it has to be assumed that the diurnal variation in serum Fe concentration does not vary significantly between any 2 d. Power calculations estimate that at a 0·05 level of significance with a power of 80 %, a two-tailed test for an experiment would require eight volunteers in a crossover design to guarantee being able to resolve a difference in absorption of 3 % between two test meals. This rises to sixteen and fifty-one volunteers for a 2 and 1 % difference in absorption respectively. The Ferrozine kit therefore appears to be inadequate for situations where an attempt is being made to resolve a small difference (< 2 %) in Fe absorption and in these cases the use of inductively coupled plasma MS or thermal ionisation MS would be expected to measure total Fe in serum with a precision (% relative SD) of less than 1 %. This would lead to an uncertainty in model absorption estimation of approximately 0·5 % and only six volunteers would be required to resolve a difference of 1 % in Fe absorption between two test meals assuming, again, that the diurnal variation in serum Fe concentration is the same on the two test meal days. Also, the Ferrozine kit requires 5 ml whole blood for triplicate analysis but less than 1 ml would be required for inductivity coupled plasma MS or thermal ionisation MS thereby reducing the total blood volume taken in a study. This would have obvious benefits in studies where the same volunteer is required to participate in more than one intervention.

The use of a single-compartment model to estimate unlabelled Fe absorption requires a larger study, with smaller doses of Fe, both haem and non-haem, to be undertaken. The 10 mg dose is higher than the quantity consumed in most meals, thus the technique must be shown to work with doses of 5 mg or less. However, the results of the present pilot study demonstrate that the estimation of the absorption of total Fe from an unlabelled source has promise and highlight the potential of mathematical modelling in nutritional studies.

Acknowledgements

This work was supported by the Food Standards Agency (formerly Ministry of Agriculture, Fisheries and Food) and the Biotechnology and Biological Sciences Research Council. The authors would like to thank Mrs Linda Oram and Mrs Diana Phillips for their skilled clinical assistance that contributed to this work.

References

Barrett JFR, Whittaker PG, Fenwick JD, Williams JG & Lind T (1994) Comparison of stable isotopes and radioisotopes in the measurement of iron absorption in healthy women. *Clin Sci (London)* **87**, 91–95.

Dale JC, Burritt MF & Zinsmeister AR (2002) Diurnal variation of serum iron, iron-binding capacity, transferrin saturation, and ferritin levels. *Am J Clin Pathol* **117**, 802–808.

Fairweather-Tait S, Fox T, Wharf SG & Eagles J (1995) The bioavailability of iron in different weaning foods and the enhancing effect of a fruit drink containing ascorbic acid. *Pediatr Res* **37**, 389–394.

Fox I, Dawson A, Loynds P, *et al.* (1993) Anticoagulant activity of Hirulog, a direct thrombin inhibitor in humans. *Thromb Haemost* **69**, 157–163.

Fox TE, Eagles J & Fairweather-Tait SJ (1998) Bioavailability of iron glycine as a fortificant in infant foods. *Am J Clin Nutr* **67**, 664–668.

Gonzalez H, Mendoza C & Viteri FE (2001) Absorption of unlabeled reduced iron of small particle size from a commercial source. A method to predict absorption of unlabeled iron compounds in humans. *Arch Latinoam Nutr* **51**, 217–224.

Hurley PJ (1975) Red cell and plasma volumes in normal adults. *J Nucl Med* **16**, 46–52.

Jacquez JA (1996) *Compartmental Analysis in Biology and Medicine*, 3rd ed. Ann Arbor, MI: BioMedware.

Lentner C (1984) *Geigy Scientific Tables*, p. 66. Basle, Switzerland: CIBA-GEIGY.

Pollycove M & Mortimer R (1961) The quantitative determination of iron kinetics and hemoglobin synthesis in human subjects. *J Clin Invest* **40**, 753–782.

Sharney L, Gevirtz NR, Wasserman LR, *et al.* (1965) Studies in iron kinetics: IV. Calculation of physiological parameters on the basis of multiple pool models. *J Mt Sinai Hosp N Y* **32**, 338–368.